

Gender-specific growth and hepatic neoplasia in medaka (*Oryzias latipes*)

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Abstract

Brief exposure of hatchling medaka (*Oryzias latipes*), to diethylnitrosamine (DEN), resulted in hepatic tumor formation in female medaka at an incidence of 2–3-fold higher than that of their male cohorts. Spontaneous liver tumor incidence was reported in unexposed 3–5 year old medaka. Gender differences were seen; higher incidence was in the females. Aspects of gender-specific growth in hatchling, immature and sexually mature control medaka are reported and studies sought to determine whether growth enhanced tumorigenesis in females. From a pool of 2000 mixed-sex, 3 week old medaka hatchlings, 1350 were exposed to an aqueous bath of 250 ppm DEN for 48 h. Another 650 hatchlings served as controls. For each fish, body- and liver-weights were recorded (BW and LW, respectively) and LW to BW ratio (hepatosomatic index—HSI) was estimated. Next, livers and carcasses were processed for histopathology. BWs of control females were significantly greater than that of males at weeks 8, 20, 32 and 44 ($P < 0.05$). LWs and HSIs were significantly greater in females versus males at all ages ($P < 0.05$). In the DEN-treated medaka, female BWs were significantly more than their male counterparts at weeks 8, 16, 20 and 32 ($P < 0.05$). Female LWs were greater than male values at all weeks except 4 and 6 ($P < 0.05$). Female HSIs were significantly greater than male HSIs at all times ($P < 0.05$). A higher incidence of foci of cellular alteration (40%) distinguished females from males (10%) at week 4 and these values reached 100% incidence (females) and 90% (males) at week 12. Tumor latency periods for adenomas and carcinomas were significantly shorter in females than in males. At week 20, the incidence of tumors was significantly higher in females than in males ($P < 0.05$). Results indicate that gender-specific differences appear in BW, but especially LW and HSI as a function of larval development, ovarian maturation and age in control and DEN-treated medaka. Tumor incidence and time to endpoint (latency period) demonstrate that female growth is a promotional-stimulus, positively modulating DEN hepatocarcinogenesis. © 1998 Elsevier Science B.V. All rights reserved.

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1. Introduction

The small aquarium fish, medaka (*Oryzias latipes*), has been shown to be sensitive to a variety of chemical carcinogens in laboratory exposures (Ishikawa et al., 1975; Hatanaka et al., 1982; Hinton et al., 1984; Hawkins et al., 1985). Studies in our laboratory have centered on the pathogenesis of hepatic neoplasia in this model, emphasizing appearance, character (Hinton et al., 1987, Laurén et al., 1990b; Teh and Hinton, 1993) and fate of foci of cellular alteration, hepatic adenomas and their emergence as hepatocellular carcinomas (Teh and Hinton, 1993).

In recent bioassays using large numbers of larvae initiated by a brief, aqueous diethylnitrosamine (DEN) exposure, tumor frequency was consistently 2–3-fold greater in female than in male medaka (unpublished observations, this laboratory). This interesting finding was the impetus for the present study which was undertaken to determine features of developmental growth in control female and male medaka from larvae through sexual maturity and through the time necessary for hepatic tumors to be expressed. Then, a study of similar duration was conducted with medaka being briefly (48 h) exposed as 21-day-old larvae to an aqueous bath of 250 ppm DEN. In this way, the entire time frame for initiation, promotion and progression of hepatic neoplasia was included enabling an evaluation of growth and its effect on the neoplastic process. Results show that preferential hepatic growth of females serves as a promotional stimulus in hepatic neoplasia and results in the female fish being at greater risk.

2. Materials and methods

2.1. Water and diet

Reconstituted water for rearing fish was prepared according to the guidelines of the United States Environmental Protection Agency (USEPA) (Horning and Weber, 1985). All tanks were connected to a partially closed recirculating system equipped with water pump, UV tube, biological and charcoal filter. Water in the recirculating system was maintained at $80\text{--}100\text{ mg l}^{-1}\text{ CaCO}_3$ (hardness), pH 8.0 ± 0.1 , $7.0 \pm 1.0\text{ mg l}^{-1}\text{ O}_2$ and $25 \pm 2.0^\circ\text{C}$. Ammonia, nitrite and nitrate were kept below detectable limits by changing the charcoal filters weekly and replacing 15–20% of system water with freshly made reconstituted water 3 \times weekly. Photoperiod was maintained at 16 h light and 8 h dark. Except for the 48 h DEN exposure, purified casein-based diet (PC) formulated in our laboratory (DeKoven et al., 1992) was fed at a daily rate of 3–4% body weight throughout the experiment.

2.2. *Animals and chemical treatments*

Three weeks after hatch, 2000 healthy larvae (21 ± 2 days old) were pooled in an 80 l tank and then separated into two groups. The first group, 1350 medaka larvae, were exposed for 48 h to an aqueous bath of 250 ppm DEN (Isopac, Sigma, St Louis, MO). DEN concentration of test water was determined at time zero, 24 h and again at 48 h, using a Shimadzu UV-VIS recording spectrophotometer UV-160. Absorbance at wavelength of 230 nm was recorded versus varying concentrations of standard.

The second group, 650 medaka, were held under similar conditions but were not exposed to DEN (control group). After exposure, fish were rinsed twice in clean, freshly-reconstituted water and then maintained in similar water in a clean 80 l tank for 1 week. After this cleansing period, 40 control and 40 DEN-treated fish were sampled for processing and the remainder were randomly assigned to one of the 22 tanks (100 fish per 40 l tank for DEN-treated and 50 fish per 20 l tank for control). Tanks designated 'control' or 'exposed' were alternated within the room to obviate position effect. Once tanks were in place and identified, control medaka were netted and randomly assigned to one of the 12 tanks. Similarly, exposed medaka were randomly assigned to one of the 10 tanks. The use of replicates of small tanks reduced sampling bias that might occur if fish were netted 'randomly' from a single large tank. To eliminate this, tanks were randomly selected at the time of each sampling and all fish in a given tank were sampled at each time period. Control tanks were sampled on the first week after exposure and each month thereafter until week 44. DEN-treated fish were sampled weekly for the first 4 weeks, biweekly for the next 4 weeks and monthly thereafter for a total of 32 weeks. All time points described in this paper will be in weeks after the 48 h exposure.

2.3. *Tissue preparation*

At necropsy, rapid external gross examination was used to estimate sex (Yamamoto, 1975) and each fish was anesthetized in 3-aminobenzoic acid ethyl ester (MS-222; 50 mg l⁻¹) and weighed. Next, livers were surgically removed, weighed and assigned to either freeze drying and vacuum embedment in glycolmethacrylate (Teh and Hinton, 1993) ($n = 50$, DEN treated; $n = 25$, control) or to fixation in 1/2 strength Karnovsky's fluid (Ito and Karnovsky, 1968) in 0.1 M phosphate buffer for transmission electron microscopic examination ($n = 50$, DEN-treated; $n = 25$, controls). HSI was established for each fish (Table 2). For BW, LW and HSI means were established for each gender at each time of sampling. To follow gonad development as a function of age and to validate gross gender identification, fish carcasses containing gonads, were fixed in 10% buffered formalin, dehydrated, cleared and embedded in paraffin. Hematoxylin and eosin stains of 6 micron thick paraffin sections were used for gonadal survey.

2.4. Liver histopathological analysis

A total of 20 livers (10 from females and 10 from males) from each sampling of exposed and control medaka were randomly selected from those animals whose livers were subjected to freeze drying and glycolmethacrylate embedment. This was continued through the 20 week sampling, the time at which 50% of the exposed population showed hepatic neoplasms. To provide complete histopathological analysis, the entire livers were serially sectioned. Sections were stained by H&E and used to detect foci of cellular alteration (FCA) (Hinton et al., 1987; Bannasch et al., 1989). Three phenotypes were identified (basophilic, clear cell or eosinophilic) using previously published criteria for medaka (Hinton, 1993; Teh and Hinton, 1993). Neoplasms were classified as hepatocellular adenoma, cholangioma, hepatocellular carcinoma, cholangiocarcinoma and mixed hepato-cholangiocellular carcinoma (Hinton, 1993). The incidence of foci and tumors at each sampling time was compared between females and males.

2.5. Statistical analysis

The two-sample Student's *t*-test was used since it enables analysis of the differences in mean values for all parameters among control and DEN groups. Significance was arbitrarily selected at $P < 0.05$. Data of foci and tumors were subjected to hypothesis testing using Mann-Whitney rank sum test. While differences may occur due to random sampling variability, the test permitted analysis of foci and neoplasms (SigmaStat, Jandel Scientific).

3. Results

3.1. DEN concentration

Concentration of DEN was within 5% of nominal concentration at 0, 24 and 48 h.

3.2. DEN toxicity

Acute DEN toxicity was seen during and for 5 days following cessation of exposure (Table 1). Mortality in DEN-treated fish over this period was 14.22% versus control mortality of 3.85% (Table 1). After fish were removed from carcinogen-bath and placed into separate individual tanks with freshly reconstituted water, subsequent mortality did not differ between groups until weeks 20 and 24. At these times, higher mortality in DEN-exposed fish was seen (Table 1). A total of 281 exposed fish died (20.8%) during the 32 weeks study, compared to 14.9% in controls (Table 1).

3.3. Growth and gender in medaka

Fish grew continuously throughout the study and growth patterns showed gender differences (Fig. 1a,b,c). When mean BW of female and male medaka were plotted, females were statistically larger than males ($P \leq 0.05$) at 8, 20, 28 and 32 weeks (Fig. 1a). Mean LW are plotted in Fig. 1b. LW of females exceeded males at each of the 10 sampling times ($P \leq 0.05$). HSI of control fish also showed gender differences (Fig. 1c). Like LW, HSI gender differences ($P \leq 0.05$) first appeared at week 4 and lasted throughout the entire 40 weeks.

Significant gender differences in DEN-exposed fish and between control and exposed fish were seen (Fig. 2a–c Fig. 3a–c Fig. 4a–f). The gender differences ($P < 0.05$) in BW were not seen until 16 weeks and again at 20 and 32 weeks (Fig. 2a). Gender differences ($P < 0.05$) in LW and HSI, prominent in young controls, were not seen in similarly aged DEN-exposed fish until week 8 and later. (Fig. 2b–c). Significantly higher values for LW and HSI characterised female fish ($P < 0.05$; Fig. 2b,c).

Statistical analyses of control and DEN-exposed fish, regardless of sex, showed treatment effects consistent with DEN toxicity (Fig. 3a–c). BW of DEN survivors was consistently lower throughout the entire experiment (Fig. 3a). In contrast, LW and HSI of DEN-exposed fish, initially decreased over first 8 weeks, were approximately two times that of control group at all later weeks except week 28. (Fig. 3b–c). While DEN-treated female medaka showed transiently diminished body weight (Fig. 4a), this parameter was further diminished in DEN-treated male medaka (Fig. 4b). Liver weight and HSI of DEN-treated females and males, initially lower at week 4, were higher at week 8 and all later weeks than their respective controls (Fig. 4c–f).

In control medaka, a 7.1-fold increase in BW was seen over 32 weeks, from an average BW of 46.5 mg at week 4, fish grew to a weight of 328.1 mg at week 32 (Fig. 3a). Liver growth progressed with body growth but at a slower rate. A 4.6-fold increase in LW occurred, from an average of 1.9 mg at week 4 to 8.9 mg at week 32 (Fig. 3b). Body, independent of liver, was not broken into its component parts. From week 4 to week 12, liver weights increased but at a rate less than the BW, resulting in a transient reduction of HSI at weeks 8 and 12 versus week 4 (Fig. 3c). Thereafter, HSI increased and was maintained at an average of 2.7%. During this same period, a 7.6-fold increase in BW was seen in DEN-treated medaka. From an average BW of 38.4 mg at week 4, fish grew to a weight of 292.3 mg at week 32 (Fig. 3a). Liver growth progressed with body growth but at a faster rate. A 10.1-fold increase in LW occurred, from an average of 1.2 to 11.7 mg at week 32 (Fig. 3b). In DEN-treated medaka, LW increased at a rate more than the BW, resulting in an increase in HSI at all weeks (Fig. 3c). HSI was maintained at an average of 3.8% from week 4 to week 32.

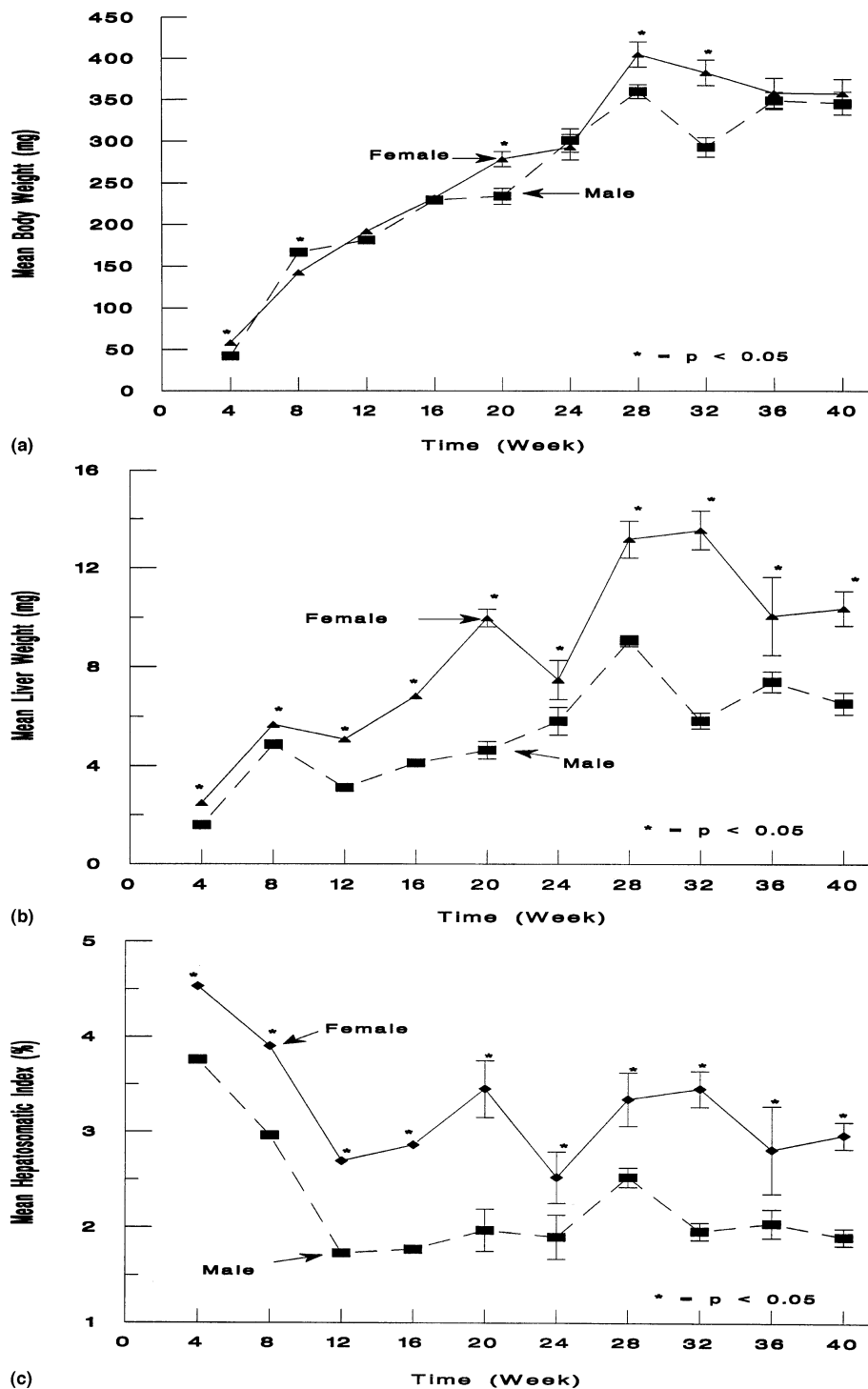


Fig. 1. (a) Control medaka, mean (± SE) body weight. (b). Control medaka, mean (± SE) liver weight. (c). Control medaka, mean (± SE) hepatosomatic index.

3.4. Morphological findings

At weeks 6 and 8, immature female and male medaka are easily identified histologically by the presence of immature oocytes (Fig. 5a,c) and primary and secondary spermatogonia (Fig. 5b,d). Medaka gonadal maturation was evidenced by presence of mature oocytes in addition to oogonia and immature oocytes (Fig. 6a) and by the presence of spermatids and spermatozoa as well as spermatogonia in testes (Fig. 6b).

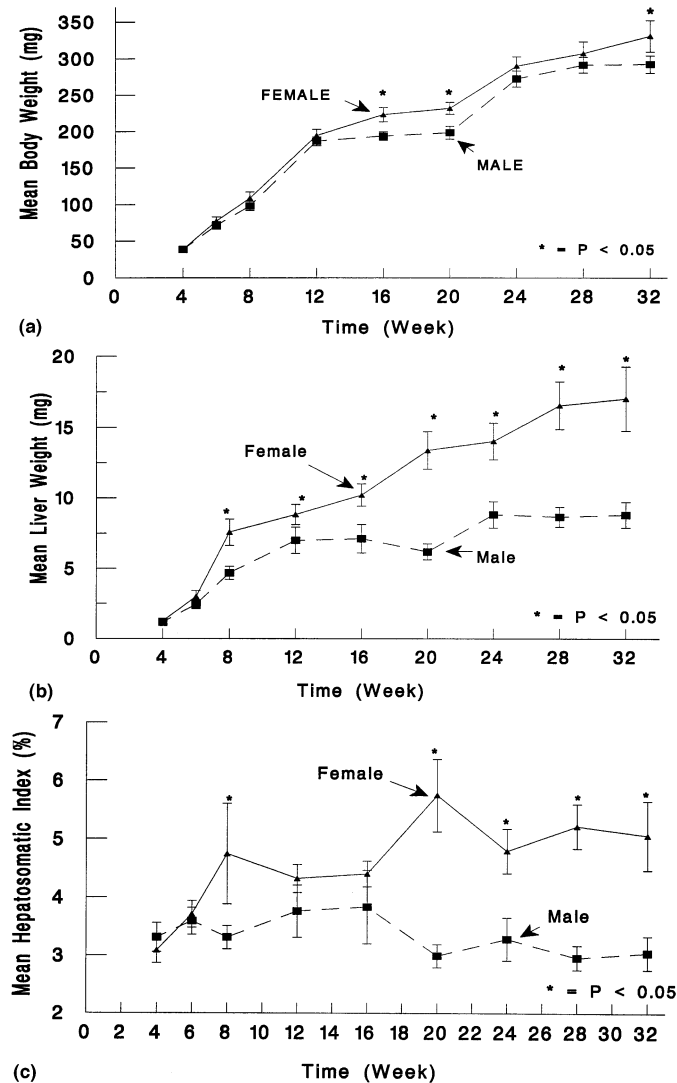


Fig. 2. DEN-treated medaka, mean (\pm SE) body weight. (b). DEN-treated medaka, mean (\pm SE) liver weight. (c). DEN-treated medaka, mean (\pm SE) hepatosomatic index.

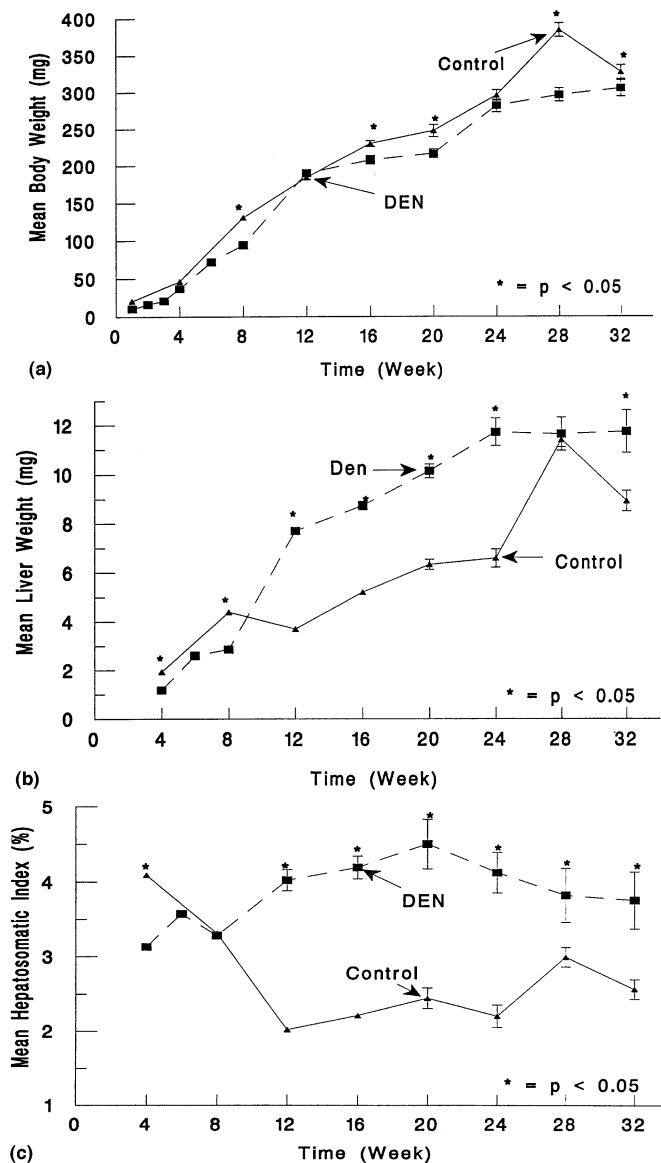


Fig. 3. Control Vs DEN-treated medaka, mean (\pm SE) body weight. (b). Control Vs DEN-treated medaka, mean (\pm SE) liver weight. (c). Control Vs DEN-treated medaka, mean (\pm SE) hepatosomatic index.

Altered hepatocellular foci (AHF) were the initial indication of a neoplastic process. In H&E-stained serial sections (Teh and Hinton, 1993), three phenotypes were seen including: clear cell, basophilic and eosinophilic. Clear cell foci appeared first (week 4) followed by basophilic and eosinophilic foci at week 6 (Table 2). In

female medaka, the number of clear cell foci peaked at week 12 with peaks for basophilic and eosinophilic foci occurring later (week 16). In males, the number of clear cell foci peaked at week 12, but basophilic and eosinophilic foci did not peak until week 20 (Table 2). The number of basophilic and clear cell foci was higher in females than in males, while that of eosinophilic foci was the reverse.

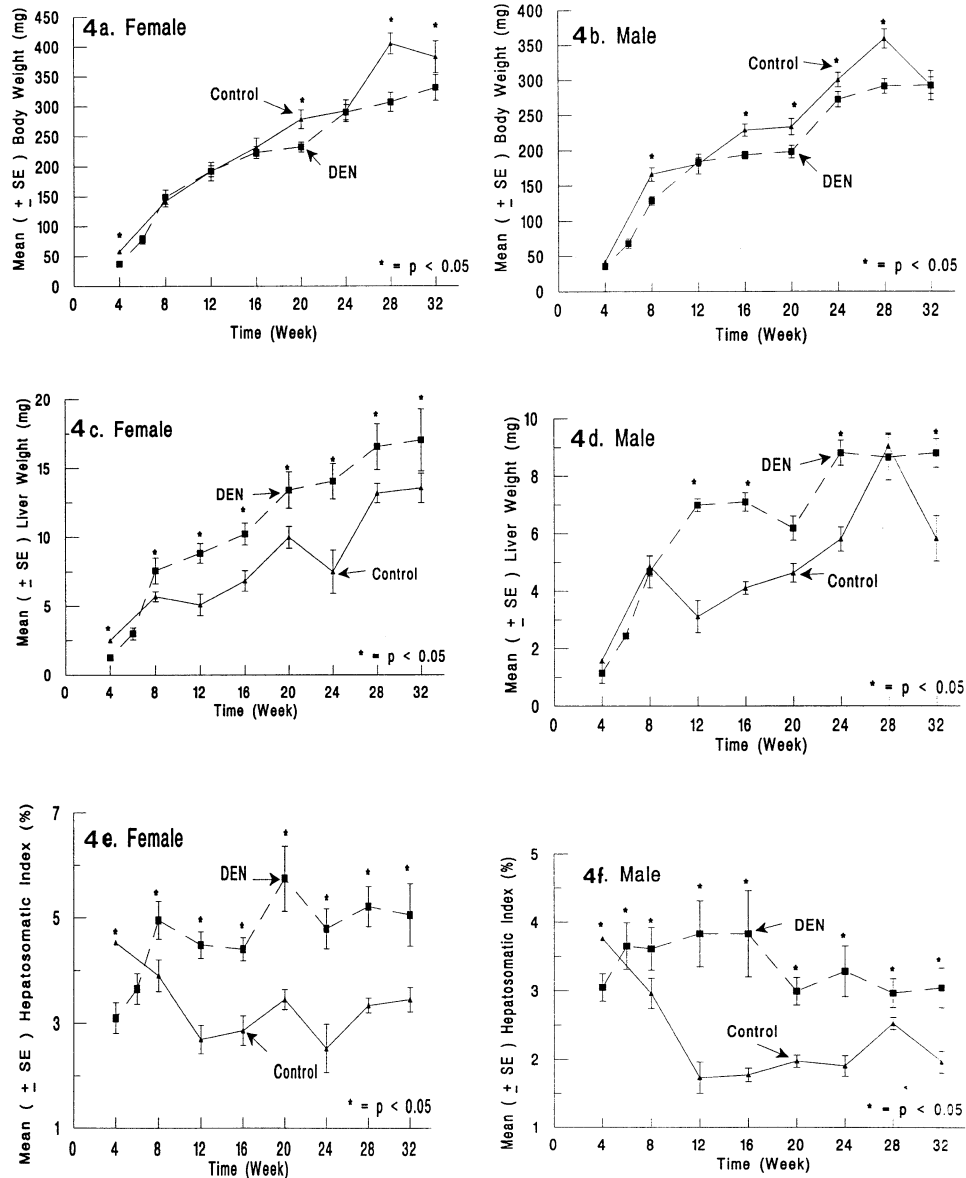


Fig. 4. Gender comparison of body weight, liver weight, and hepatosomatic index of control and DEN-treated medaka.

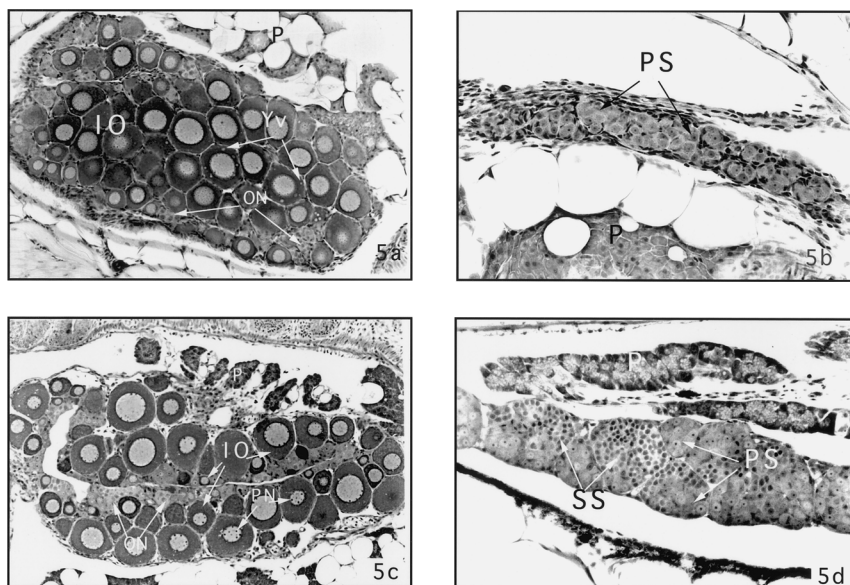


Fig. 5. Gonads of 6- and 8 week old medaka. (a). Section of immature ovary, 6 week old medaka. Abundant immature oocytes (IO) and a few undifferentiated oogonia nests (ON) are seen. P, pancreas; YV, yolk vacuoles; H&E. $125\times$. (b). Section of immature testis, 6 week old medaka. Primary spermatogonia (PS), only, are seen. P, pancreas. H&E. $200\times$. (c). Section of immature ovary, 8 week old medaka. Increased number of immature oocytes (IO) with provitelline nucleoli (PN) are seen. P, pancreas. H&E. $125\times$. (d). Section of immature testis, 8 wk old medaka. In this section, approximately one half were primary (PS) and one half were secondary spermatogonia (SS). P, pancreas. H&E. $200\times$.

Total AHF frequency increased as a function of time after carcinogen exposure. All types of AHF were observed in both sexes. While 40% of female and 10% of male medaka had AHF at week 4, the incidence reached 100% for females at week 12 but not until week 20 for males (Table 3). Except for basophilic cell phenotypes, which showed gender differences at weeks 12 and 16 ($P < 0.05$), no gender differences were encountered in total AHF and in numbers of eosinophilic or clear cell foci (Table 3).

Three hepatocellular adenomas and one carcinoma were first seen in female medaka at week 12. The first hepatocellular carcinoma in males occurred in the week 20 sample (Table 2). At week 20, 70% of the females and 10% of the males examined had neoplasms (Table 3). In addition to the hepatocellular neoplasms, cholangiomas, cholangiocarcinomas and mixed cholangio-hepatocellular carcinoma were also observed. For consistency, despite their relatively low abundance, latency periods for these neoplasms are presented as well (Tables 2 and 3). Gender differences in tumor production were seen at week 20. The incidence of tumors was significantly higher in female than in male medaka ($P < 0.05$).

4. Discussion

Several aspects of medaka growth proved interesting in this study. Medaka, like other fish, apparently grow continuously if food and free space are not limiting (Moyle and Cech, 1982; Weatherley and Gill, 1987; Wieser, 1993). The most rapid medaka growth occurred between week 4 and 12 of this study (Figs. 1 and 2a). This

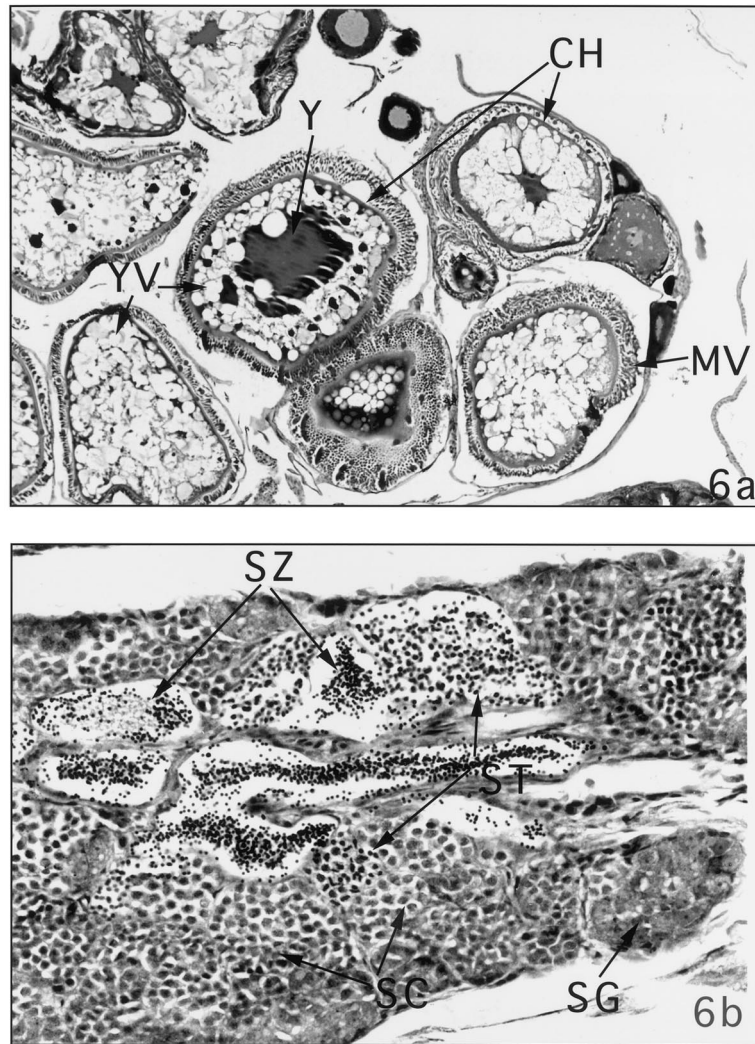


Fig. 6. Gonads of 12 week old medaka. (a). Section of mature ovary, 12 week old medaka. Note marked increase in yolk vacuoles (YV) of cytoplasm and a distinct chorion (CH) are shown. Y, yolk; MV, microvilli. H&E. $540\times$. (b). Section of mature testis, 12 wk old medaka. Various stages of spermatogenesis are shown. SG, spermatogonia; SC, spermatocytes; ST, spermatids; and SZ, spermatozoa. H&E. $540\times$.

Table 1
Sampling schedule and mortality data of control and DEN-treated medaka

Weeks	Control				DEN			
	# Of fish	# Of fish died	% Of fish died ^a	Total # dead fish	# Of fish	# Of fish died	% Of fish died ^a	Total
1	650	25	3.85	25	1350	192	14.22	192
4	550	3	0.55	28	900	5	0.56	197
8	500	5	1.00	33	700	11	1.57	208
12	450	15	3.33	48	600	13	2.17	221
16	400	14	3.50	52	500	14	2.80	235
20	350	4	1.14	56	400	15	3.75	250
24	300	9	3.00	65	300	14	4.67	264
28	250	13	5.20	78	200	9	4.50	273
32	200	19	9.50	97	100	8	8.00	281

Tanks were randomly selected using a random number table.

^a % of fish died was derived by dividing the number of fish that died by the number of fish present at the particular sampling time.

corresponds with reports of maximal growth in other fish (Moyle and Cech, 1982). Although not individually determined, gonad weight, by retrospect, would have been an important factor to monitor. Dynamics of female liver growth and its relationship to gonadal maturation are under investigation currently and will be the subject of a future report. Medaka under our culture conditions first reach reproductive activity at ≈ 10 weeks. This was shown by the presence of oogonia and oocytes in ovaries of this study (Fig. 6a) and spermatids and spermatozoa in testes (Fig. 6b). From week 4 to 12, liver weights increased but at a rate less than the BWT, resulting in a transient reduction of HSI at weeks 8 and 12 versus week 4 (Fig. 1c). Thereafter, HSI increased and was maintained at an average of 2.4%. Perhaps gonadal growth, not measured in this study, would explain the transient reduction in HSI.

Photoperiod, temperature and other factors affect growth rates and may act through variations in growth hormone secretions (Donaldson et al., 1979). Photoperiod and temperature however were constant factors in the present study. Therefore, the gender differences in BWT, LWT and HSI were likely related to gonadal weight and hormonal stimulation. The mature testes and ovaries can account for 12–70% of the total body weight with annual breeders showing relatively higher values (Moyle and Cech, 1982, Munro et al., 1990). Liver growth in fish is, to an extent, related to ovarian growth. The liver is the site for vitellogenin synthesis in females (Rankin and Jensen, 1993; Jobling, 1995) and for the egg envelope precursor protein, choriogenin (Murata et al., 1997). Oocyte growth occurs by uptake of circulating vitellogenin, which is then modified by and deposited as yolk in the oocyte (Wallace, 1978; Wallace and Selman, 1990). On the other hand, no or minimal involvement of the liver is needed for male gonadal development and function (Rankin and Jensen, 1993). We postulate that differences in the role of liver during female gonadal growth and endogenous, as well as exogenous vitellogenesis (van Bohemen et al., 1981a,b), were major factors in female LWT and HSI. Female mean values for these parameters were 1.64- and 1.41-fold higher than those of males (Fig. 1b,c).

Physiological and morphological studies using rainbow trout (*Salmo gairdneri*, now *Oncorhynchus mykiss*), described a strong link between gonadal development and liver growth (Olivereau and Olivereau, 1979; van Bohemen et al., 1981a,b; Selman and Wallace, 1983; Ng et al., 1984; Hampton et al., 1989). In those studies, higher HSI values and numbers of hepatocytes occurred as a result of endogenous and exogenous vitellogenesis (van Bohemen et al., 1981a,b, 1982; Hampton et al., 1989). For annual breeders such as trout, gonadal development can be considered to consist of a series of interrelated phases, separated by time. The involvement of liver during vitellogenesis only occurred in the beginning of yolk production (van Bohemen et al., 1981a,b). Medaka, on the other hand, are seasonal breeders (Grady et al., 1991) and under our laboratory culture conditions, can produce eggs all year long. Therefore, the stage is set for continual production of estrogen and for hepatotrophic effects. The higher LWT and HSI values of control females throughout this study may be attributed to this process.

Table 2
Gender differences in number of altered hepatocellular and neoplasms using 2-dimensional analysis

Week ^a	Sex ^b	# Fish	Total number of AHF or neoplasms					Any type	Hepatocellularadenoma	Cholangioma	Hepcarcinoma	CholangioCA	MixedCA
			Basophilic	Clear cell	Eosinophilic								
4	F	10	0	8	0	8	0	0	0	0	0	0	0
	M	10	0	1	0	1	0	0	0	0	0	0	0
6	F	10	8	16	1	25	0	0	0	0	0	0	0
	M	10	2	8	1	11	0	0	0	0	0	0	0
8	F	10	10	8	2	20	0	0	0	0	0	0	0
	M	10	4	3	3	10	0	0	0	0	0	0	0
12	F	10	35*	29	3	67	3	0	0	1	0	0	0
	M	10	5*	28	8	41	0	0	0	0	0	0	0
16	F	10	46*	14	9	69	1	0	0	3	1	0	0
	M	10	15*	9	4	28	0	1	0	0	0	1	0
20	F	10	14	20	8	42	4	2	2	3	0	0	0
	M	10	8	18	17	43	0	0	0	1	0	0	0

^a Week, Weeks after onset of exposure; Hep, hepatocellular; CA, carcinoma.

^b Sex: F, female; M, male.

* Significant differences between female and male ($p < 0.05$), according to Mann-Whitney's test.

Table 3
Gender differences in incidences of medaka with liver foci and neoplasms

Week ^a	Sex ^b	# Fish	Incidence (%) of medaka with foci or neoplasms									
			Baso	Clear	Eosino	Any type	HepA	ChoA	HepCA	ChoCA	MixedCA	Any type
4	F	10	0	40	0	40	0	0	0	0	0	0
	M	10	0	10	0	10	0	0	0	0	0	0
6	F	10	40	70	10	80	0	0	0	0	0	0
	M	10	20	50	10	60	0	0	0	0	0	0
8	F	10	40	50	20	70	0	0	0	0	0	0
	M	10	30	20	30	40	0	0	0	0	0	0
12	F	10	100*	80	10	100	20	0	10	0	0	20
	M	10	40*	90	30	90	0	0	0	0	0	0
16	F	10	90*	60	20	90	10	0	20	10	0	40
	M	10	50*	40	40	70	0	10	0	0	10	20
20	F	10	70	60	90	100	30	20	30	0	0	70*
	M	10	50	80	80	100	0	0	10	0	0	10*

^a Week, weeks after onset of exposure.

^b Sex: F, female; M, male; Baso, basophilic foci; clear, clear cell foci; eosino, eosinophilic foci; HepA, hepatocellular adenoma; ChoA, cholangioma; HepCA, hepatocellular carcinoma; ChoCA, cholangiocarcinoma; MixedCA, mixed cholangio-hepatocellular carcinoma.

* = Significant differences between female and male ($P < 0.05$), according to Mann-Whitney's test.

Data presented in this study clearly show that both control and DEN-treated LWT and HSI are significantly higher with age in female than in male medaka (Fig. 1b–c; Fig. 2b–c). Unpublished studies from this laboratory using morphometric analysis on serial sections of entire normal liver showed female hepatocytes were $\approx 25\%$ smaller, but 200% more numerous than male hepatocytes at week 12. Other mammalian studies reported liver growth and increased hepatic function as a direct response to endogenous and exogenous estrogens (Ochs et al., 1986; Schulte-Hermann et al., 1988). Therefore, an increase in number of hepatocytes with a concomitant increase in HSI, suggests that the increased LWT might be due to hepatocyte proliferation. The female medaka liver growth with high LWT and HSI, of the present study, may be a response to endogenous estrogens and/or other hepatotrophic factors. It will be interesting to initiate fish at different weeks and to determine whether growth bursts will promote tumorigenesis.

Gender differences are seen in incidence of spontaneous neoplasms in mice (Maita et al., 1988; Tamano et al., 1988) and in medaka (Masahito et al., 1988). The finding of more spontaneous tumors in female than male medaka suggests that steroid hormones may play an important role in tumor production. Vesselinovitch (1987) demonstrated that gonadectomy retarded hepatic tumor emergence. Interestingly, Weghorst et al., 1989 found that when DEN initiated mice were given phenobarbital treatment later, female hepatic tumorigenesis was increased, while that of the males was suppressed. Gender differences, with respect to carcinogen initiation, could explain the tumor incidences of the present study. However, gender specific differences in DEN metabolism and in DNA adduct formation have not been investigated in medaka. Until these aspects are quantitatively established for each gender, we do not know whether earlier events in carcinogenesis affect eventual tumor incidence as well as those involved in promotion of initiated cells into foci and tumors.

The present study took advantage of a single lobed, small liver ($2 \times 2 \times 3 \text{ mm}^3$, weighing $\approx 10 \text{ mg}$ in adults) which has proven excellent for reliable serial morphologic and stereologic analyses with reasonable cost and time. Enumerations of foci and tumors reflected the entire liver of each animal. This omitted the problems inherent with distribution and size of foci seen in two-dimensional studies (Harada et al., 1989; Morris, 1989; Pitot et al., 1989). We observed foci that extended up to $200 \mu\text{m}$ in thickness. Basophilic foci, on early sections, were subsequently—on deeper sections—diagnosed as an adenoma or carcinoma. Finally, the incidences of foci in this study decreased as tumor incidence increased. This is a common event in rodent hepatocarcinogenesis and signifies that at least some of the former become the latter.

DEN toxicity was targeted at the liver and resulted in low LWT and HSI initially; but the strong hepatotrophic stimulus in the females pushed liver growth beyond that of the normal females. In DEN-treated males, toxicity targeted BWT. Although the extent of liver growth in DEN-treated males proved lower than that of DEN-treated females, it was consistently higher than values for controls of either gender.

The mortalities in the control group at 44 weeks were unexpected. In one of our ongoing studies, using the same protocol, we have found that systemic mycobacteriosis developed and was more severe in controls than in DEN-treated medaka. We maintain veterinary laboratory animal surveillance on our colony and will report on pathologic conditions in untreated medaka in a future publication. However, the timing of the mortalities and the absence of non-neoplastic pathologies in medaka from 3–32 weeks lead us to believe that results of this study were not affected by this condition.

In conclusion, female medaka showed increased hepatic growth over their male cohorts. When initiated as hatchlings by 48 h exposure to the medaka hepatocarcinogen, DEN, females developed more foci and tumors and did so more rapidly than males. In this model, gender specific growth characteristics appear to promote DEN initiated hepatocarcinogenesis.

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